Charles University in Prague
First Faculty of Medicine

Institute of Physiology
Academy of Sciences of the Czech Republic

Structure and function of the vanilloid receptor TRPV1

Klára Sušánková

Summary of PhD Thesis

Prague 2006

PhD thesis was elaborated at the Department of Cellular Neurophysiology of the Institute of Physiology, Academy of Science of the Czech Republic, Prague. It was a part of the PhD studies of Biomedicine at the 1st Faculty of Medicine, Charles University in Prague, with specialization for Neuroscience.

Applicant: Klara Susankova

Address: Institute of Physiology AS CR
Videnska 1083
142 20 Prague 4
Czech Republic
phone: 00420 29644 2759
fax: 00420 29644 2488
email: susinka@biomed.cas.cz

Commission: Neuroscience

Supervisor: Viktorie Vlachová, RNDr., D.Sc.

Consultants: Ladislav Vyklický Sr., M.D., D.Sc.
Jan Teisinger, Ing., Ph.D.

Opponents:

Summary was sent out on:

Defence of the Thesis:

Chairman of the Commission:

Table of contents:

1. Summary

The vanilloid receptor (TRPV1) plays a role of the polymodal detector of nociceptive stimuli in a subset of small and medium sized primary sensory neurons in mammals. This channel can be activated by vanilloid compounds, low pH, and noxious heat, and its function is modulated by a wide range of endogenous and exogenous agents.

Our results demonstrate that the reducing agent dithiothreitol (DTT) strongly potentiates both the native and recombinant rat TRPV1 channel when applied at millimolar concentrations to the external solution under intact whole-cell conditions. Since the effects of DTT were almost immediate, dose-dependent, and reversible, the contribution of extracellular cysteine residues within the putative pore-loop region of TRPV1, Cys616, Cys621, and Cys634 has been proposed. We show that the chemical modification of TRPV1 by both reducing and oxidizing agents leads to an increased response to heat. In addition, we identify the extracellularly located cysteine at position 621 which contributes to the DTT-induced potentiation of heat-activated currents mediated by TRPV1. Our data also indicate that capsaicin concentration is radically altered by the presence of oxidizing agents. More generally, the redox-active substances can substantially affect the activity of TRPV1 channels by influencing their modulators or coactivators.

We further demonstrate that copper-o-phenanthroline (Cu:Phe), well known as an oxidizing reagent, acts as a channel blocker of the TRPV1 channels expressed in HEK293T cells and of the native capsaicin receptors expressed in rat DRG neurons. We show that the complex Cu-o-phenanthroline blocked the TRPV1 channel in a concentration dependent manner. The blocking effect of Cu:Phe was reversible, voltage-dependent, and did not involve extracellularly located cysteines.

Our results indicate that the intracellular C-terminal tail of the vanilloid receptor in HEK293T cells is required for channel activation. The truncation of 31 residues was sufficient to induce changes in functional properties of TRPV1 channel. More pronounced effects of C-terminal truncation were seen in mutants lacking 72 aa. These changes were characterized by a decline of capsaicin-, low pH-, and heat-sensitivity. Truncation of the entire TRPV1 C-terminal domain (155 residues) resulted in a nonfunctional channel.

2. Introduction

Vanilloid receptor 1 [transient receptor potential (TRP)V1, formerly VR1] has been suggested to function as a multimodal signal transducer of noxious stimuli in the mammalian somatosensory system (Caterina et al. 1997). Noxious thermal stimuli (< 43 °C), acidic pH (< 6.8), or the alkaloid irritant capsaicin are required to open the TRPV1 channel. At room temperature and pH of 7.3, TRPV1 behaves as a voltage-gated outwardly rectifying nonselective cation channel, because it can be activated strongly by depolarizing voltage steps in the absence of any agonist (Chuang et al. 2001; Reeve and Bevan 2002; Vlachova et al. 2002). The molecular structure of TRPV1 was identified by (Caterina et al. 1997) as a protein consisting of 838 amino acids with six transmembrane segments, a pore-forming loop between TM5 and TM6 and with the N- and C- terminals located intracellularly. Apparently four identical subunits form the functional nonselective cation channel (Jahnel et al. 2001; Kedei et al. 2001; Kuzhikandathil et al. 2001).

Although much knowledge of the structure and function of the TRPV channel subfamily has accumulated recently (for review, see Nilius and Voets 2005; Pedersen et al. 2005; Ramsey et al. 2006), the critical structural domains and the mechanisms by which various external stimuli translate into channel gating remain poorly understood. A TRPV1 variant, the stretch-inactivated channel (SIC), has a truncated N-terminal domain and shares identical transmembrane spanning domains with TRPV1 (Schumacher et al. 2000b). Another TRPV1 variant, the VR 5‘ splice variant, which is also insensitive to vanilloids, low pH, and thermal stimuli, encodes a C-terminal domain identical to TRPV1 but lacks the major part of the N terminal (Schumacher et al. 2000a). It has been suggested that the C-terminal domains of the TRP receptors act to ensure the uniqueness of their function (Xue et al. 2001). In the predicted TRPV1 C terminal, a serine residue S800 that can be directly phosphorylated by protein kinase C (Numazaki et al. 2002) and a putative Walker-type nucleotide-binding site (L726-W740) have been identified to date (Kwak et al. 2000). However, how the C-terminal domain contributes to the conformational stability of TRPV1 channel and the extent to which it influences its function still remain to be determined.

The function of the vanilloid receptor is modulated by a wide range of endogenous and exogenous agents (for review, see Planells-Cases et al. 2005). Among them, redox

agents have previously been shown to influence ligand binding to the capsaicin receptor (Szallasi et al. 1993; Szallasi et al. 1995). TRPV1 contains 18 cysteine residues of which three C616, C621 and C634 are located on the extracellular side of the receptor, in the pore-forming loop and the region flanking it. The existence of these cysteine residues of TRPV1 prompted us to explore whether the activity of the vanilloid receptor can be regulated by redox-active substances. In an attempt to identify the mechanisms involved in the redox modulation of TRPV1 channel, the effects of different oxidizing and reducing agents on capsaicin- and heat-induced membrane currents were studied in HEK293T cells expressing rat TRPV1.

3. Experimental questions

The general objective of the present study was to characterize the cellular mechanisms of modulation of the vanilloid receptor and describe how is the molecular structure related to the function of the TRPV1 receptor.

The specific aims of our experiments were to:

1. Describe the effects of reducing and oxidizing agents on TRPV1 activation and identify amino acid residues that participate in this type of modulation.

2. Determine the mechanism of TRPV1 inhibition by an oxidizing reagent copper-o-phenanthroline.

3. Functionally characterize the C-terminal region of the vanilloid receptor by using site directed mutagenesis and homology modeling, with a focus on its contribution to the thermal sensitivity of TRPV1 channel.

4. Methods

Preparation of cell cultures:

DRG culture of the rat

Primary cultures of dorsal root ganglion (DRG) neurons were prepared in two steps. The experiments were carried out according to the guidelines of the European Community's Council Directive and with approval of the Institutional Animal Care and Use Committee. Two- to three-day-old Wistar rats were anaesthetized with ether and decapitated. Hippocampi were removed, dissociated with trypsin and plated on collagen-coated glass coverslips in a nutrient medium composed of 90% Eagle's minimum essential medium (MEM) and 10% fetal bovine serum. When the glial cultures became confluent, usually after 6-9 days, the medium was switched to nutrient-supplemented MEM with 5-fluoro-2-deoxyuridine (15 mg ml^{-1}), uridine (35 mg ml^{-1}) and 10% horse serum to minimize cell division. In the second step, we took 2- to 3-day-old rats of the same breed and killed them according to the same protocol as mentioned above. Afterwards we dissected dorsal root ganglia and incubated them at 37 °C in phosphate-buffered saline (PBS) containing 2% collagenase for 45 min and then in PBS with 0.3% trypsin for 10 min. The ganglia were then rinsed with calcium- and magnesium-free PBS and dissociated by trituration using a fire-polished Pasteur pipette. DRG cells were plated on the glial feeder layer cultures, grown in a medium composed of 90% MEM and 10% fetal calf serum and maintained at 37 °C in a water-saturated atmosphere with 5% CO_2. The next day the medium was replaced by a nutrient-supplemented MEM containing 10% horse serum and 5-fluoro-2-deoxyuridine (15 mg ml^{-1}) and uridine (35 mg ml^{-1}). Nerve growth factor (mNGF 2.5S; Alomone Laboratories, Israel; 30 ng ml^{-1}) was added to the nutrient medium, which was changed twice a week.

Transfection and maintenance of HEK293T cells

HEK293T cells (SD 3515; American Type Culture Collection, Manassas, VA) were cultured in OPTI-MEM I (Invitrogen, Paisley, Scotland) supplemented with 5% fetal bovine serum. Cells were plated on to dishes coated with collagen

at a density ~180,000 cells cm^{-2}. 293T cells were transfected transiently with 300 to 400 ng/dish recombinant plasmid DNA encoding wild-type or mutant rat TRPV1 in pcDNA3 vector (wild-type kindly provided by D. Julius, San Francisco, CA) using either the LipofectAMINE 2000 (Invitrogen) or Magnet-assisted transfection (IBA GmbH, Göttingen, Germany) method according to the manufacturers' protocols. To identify the transfected cells in the electrophysiological experiments, DNA plasmid encoding green fluorescent protein (GFP) in pQBI 25 vector (TaKaRa, Kyoto, Japan) was cotransfected at a concentration of 400 ng/dish. Transfected cells were replated onto glass coverslips (three 12-mm coverslips per 35-mm dish) coated with collagen and poly-L-lysine. Electrophysiological experiments were performed 24 to 48 h after transfection. For each experimental group, five to eight GFP-positive cells per coverslip were studied from at least three different (independent) transfections.

Constructions of mutants of TRPV1 receptor:

Rat TRPV1 mutant were constructed by PCR amplification using rat TRPV1-specific overlapping primers synthesized to contain a point mutation converting the respective nucleotides at wanted positions. The QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used according to the manufacturer's protocol to perform point mutations in TRPV1. All site-directed mutated constructs were confirmed by DNA sequencing using an automated sequencer (ABI PRISM 3100; Applied Biosystems, Foster City, CA).

Immunohistochemistry:

Polyclonal anti-TRPV1 raised against the peptide corresponding to the N terminal of rat TRPV1 (Affinity BioReagents, Golden, CO) was diluted 1:1000 in PBS. FITC-conjugated donkey antirabbit IgG (Abcam, Cambridge, UK) was used in a 1:100 dilution in PBS as the secondary antibody. Before seeding and transfection of the cells, three coverslips were placed in the culture dish. Twenty-four hours after transfection, the cells on the coverslips were washed once with PBS, fixed with 4% paraformaldehyde for 30 min, and permeabilized with 0.5% Triton X-100 in PBS for 5 min. After permeabilization, the cells were washed three times for 10 min in PBS and then incubated for 30 min in PBS

solution with 0.25% BSA and 0.25% gelatin to eliminate nonspecific binding. Cells were incubated overnight at 4 °C with primary antibody, washed three times in PBS, incubated with secondary antibody for 1 hour, and washed again three times in PBS. Immunostained HEK293T cells were visualized using confocal laser scanning microscopy (MRC 600; Bio-Rad, Hercules, CA) attached to an inverted microscope (Nikon Diaphot; Nikon, Tokyo, Japan).

Electrophysiology:

Whole-cell membrane currents were recorded using an Axopatch-1D amplifier and pClamp8 software (Axon Instruments, Foster City, CA). Electrodes were pulled from borosilicate glass; after fire polishing and filling, they had a resistance of 2-4MΩ. The series resistance was usually < 10 MΩ and was compensated to ~80%. A system for fast superfusion of the neurons was used for drug and heat application. It consisted of a manifold of seven fused silica capillaries connected to a common outlet made from a glass capillary around which insulated copper wire (20 μm thick) was coiled to pass direct current for heating the solutions superfusing the neuron under investigation (Dittert et al. 1998). The temperature of the superfusing solution was measured by a miniature thermocouple inserted into the outlet capillary near its orifice that was placed < 100 μm from the cell under investigation. Before and after the test solutions, the cells were superfused with control extracellular solution of the following composition (in mM): 160 NaCl, 2.5 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 HEPES, and 10 glucose, pH adjusted to 7.3 with NaOH. The intracellular pipette solution contained (in mM): 125 Cs-gluconate, 15 CsCl, 5 EGTA, 10 HEPES, 2 NaCl, and 0.5 $CaCl_2$, pH adjusted to 7.3 with CsOH. Capsaicin was dissolved in 100 μl of DMSO and diluted with 0.9 ml of distilled water to make a stock solution of 1mM.

Drug application:

The oxidizing reagent, Cu(II)-1,10-phenanthroline, was freshly prepared prior to each experiment and used within 4 h at room temperature. $CuSO_4$ was solubilized in H_2O (200 mM stock solution) and mixed with 1, 10-phenanthroline (Sigma; 200 mM stock solution in ethanol). The final concentrations of copper and

1, 10-phenanthroline in the bathing solution were mixed in a molar ratio of 1:4 ($CuSO_4$: 1, 10-phenanthroline). The final concentration of ethanol was < 0.001%. DTT, H_2O_2, glutathione (GSH), and diamide were prepared from a stock solution of 1, 10, and 1 M, respectively, in distilled water; the final dilutions were used for about 8 h. *N*-Ethylmaleimide (NEM) was freshly prepared before each experiment from a stock solution of 2 M in ethanol. The final concentration of ethanol was < 0.05%. DTNB was diluted directly into the bath solution to achieve the final concentration. The experimental extracellular solutions containing the redox reagents were prepared, and the pH was adjusted immediately before use and checked after the experiments.

Molecular modeling:

The C-terminal sequence from 690 to 838 was aligned with the known x-ray structure of the fragile histidine triad (FHIT) protein, extracted from the Protein Data Bank (entry 1FIT; http://www.rcsb.org/pdb/). The primary structure of this protein shows a high degree of similarity (44%) with the TRPV1 C terminal. The primary structures were aligned with the template structures by ClustalX (Thompson et al. 1997) and adjusted manually when necessary. Three-dimensional models of the C tail constituted by all nonhydrogen atoms were generated by the Modeller 6 package (Sali and Blundell 1993). All the obtained models were subjected to a short simulated annealing refinement protocol available in Modeller. Finally, the tertiary structure models were checked with Procheck (Laskowski et al. 1993).

Statictical analysis:

All data are expressed as means ± SEM. Overall statistical significance was determined by ANOVA. In cases of significance ($p < 0.05$), statistical comparisons were performed by Student's *t* test for individual groups. A rectification index (RI) of voltage-gated TRPV1-mediated whole-cell membrane currents was calculated using the following equation: $RI = [I_{+60}/(60 - V_{rev})]/[I_{-40}/(-40-V_{rev})]$. Here, I_{+60} and I_{-40} are the steady-state amplitudes of the responses recorded at holding potentials of +60 and -40 mV, and V_{rev} is the estimated reversal potential. The temperature coefficient Q_{10} was used to characterize the temperature dependence

of the membrane current (Vyklický et al. 1999). Data sampled at the rising phase of the temperature ramp were pooled every 0.5 °C. The absolute current values were plotted on a log scale against the reciprocal of the absolute temperature (Arrhenius plot). Q_{10} values were determined by using the following formula: $Q_{10} = \exp[\Delta T \times Ea/(R \times T1 \times T2)]$, where R is the gas constant, ΔT =10 Kelvin, and Ea is an apparent activation energy estimated from the slope of the Arrhenius plot between absolute temperatures $T1$ and $T2$. Dose-response data were fitted to the Hill equation, as follows: $I = I_{max}[(\text{capsaicin})^n/([\text{capsaicin}]^n + EC_{50}{}^n)]$, where I is the normalized current at a given concentration of capsaicin, I_{max} is the maximal current, EC_{50} is the concentration of half-maximal activation, and n is the Hill coefficient.

5. Results

5.1. Reducing and oxidizing agents sensitize heat-activated current of the vanilloid receptor TRPV1

Our results demonstrate that the reducing agent dithiothreitol (DTT) reversibly, and in a concentration dependent manner, facilitates membrane currents induced by noxious heat and by capsaicin in DRG neurons isolated from rat as well as in rat TRPV1-transfected HEK293 cells. In DRG neurons, the membrane currents induced at 45 °C were increased significantly in the presence of 30 mM DTT by 2.5 ± 0.5 (paired t-test, $p < 0.0001$; $n = 15$). In TRPV1-HEK293 cells I_{HEAT} induced at 45 °C elevated from 654 ± 213 pA to 891 ± 293 pA by addition of 2 mM DTT which is 1.5-fold increase (1.5 ± 0.2; paired t-test, $p < 0.001$; $n = 19$). DTT also shifted the threshold to lower temperature, 34 °C and 40 °C, in the DRG and in the TRPV1-transfected cell, respectively (Fig. 1). Mock cells marked only with GFP are completely insensitive to noxious heat, capsaicin and combination of these two algogenic agents, indicates that facilitatory effects of DTT are exerted on TRPV1 receptor and not on other ion channels expressed in DRG neurons.

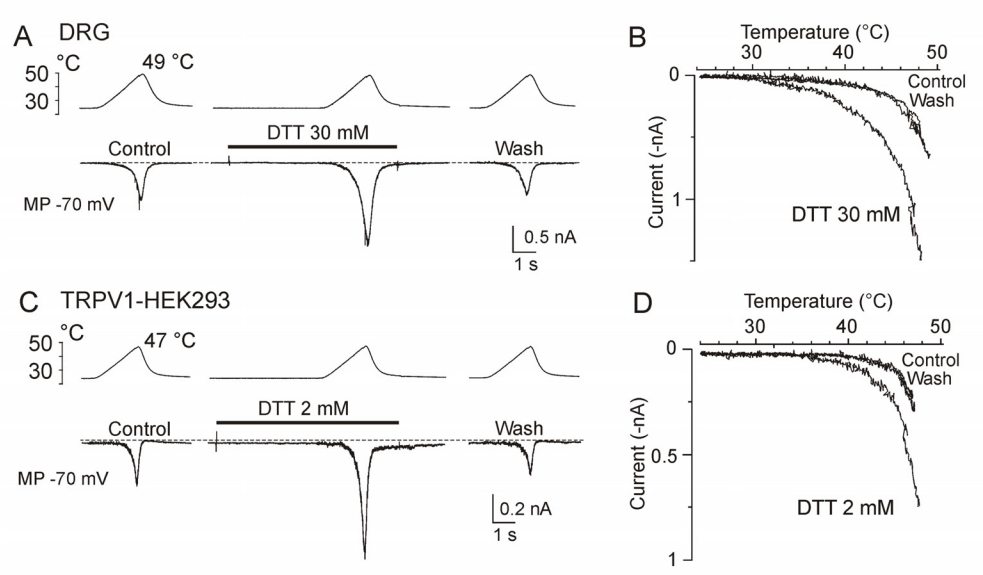

Figure 1. DTT increases membrane current induced by noxious heat. (A) Membrane currents induced by 3 sec ramps of increasing temperature of the superfusing solution to 49 °C in a cultured DRG neuron. The temperature applied is shown above the records. DTT (30 mM) was applied for the time indicated by the bar above the record. **(B)** The diagram shows current-temperature relationships in the control, in DTT and after washing. **(C, D)** The same as in (A, B) in a HEK293 cell transfected with rat TRPV1, the concentration of DTT was 2 mM. The cells were clamped at a membrane potential (MP) of -70 mV.

Since the effects of DTT were almost immediate, dose-dependent and reversible, the contribution of extracellular cysteine residues within the pore-loop region of TRPV1, C616, C621 and C634 has been proposed. We substituted these residues with glycine either individually or as a triple mutant (3CYS) and tested the effects of redox-active substances on the whole-cell membrane currents induced by heat.

In the presence of DTT (10 mM), heat-activated currents were potentiated in all constructs over the temperature range of 37-47 °C; however this effect was significantly reduced in the C621G and 3CYS ($p < 0.05$; $n = 11$ and 16) (see Fig. 2). These finding suggest that the cysteine residue at position 621 is included in the modulation of the TRPV1 channel by the DTT on the extracellular side. The 3CYS mutation did not fully abolish the potentiating effects induced by DTT. We exclude the possibility that some effects of DTT on 3CYS might be caused by chelation of trace amount of divalent cations and we also made an attempt to exclude the involvement of protein kinase C (and perhaps also PKA or calcium/calmodulin-activated protein kinase II) activation by DTT. The remaining sensitivity of 3CYS to DTT observed in our experiments could thus be unrelated to thiol-disulfide exchange (Alliegro 2000).

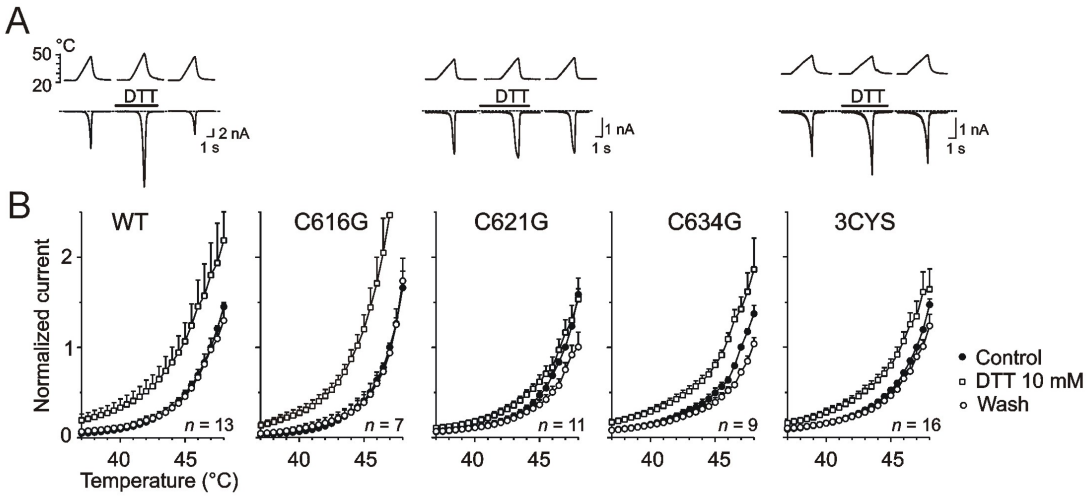

Figure 2. DTT-induced potentiation of heat-evoked currents reduced in C621G and 3CYS mutants of heterologously expressed rat TRPV1. (A) Representative families of whole-cell currents evoked in wild-type, C621G, and 3CYS mutants by 3 sec ramps of heated solutions from 24-48°C in extracellular control solution, in the presence of 10 mM DTT, and recovery 1 min later. **(B)** Average heat-induced currents were normalized to currents evoked at 47 °C in extracellular control solution shown with error bars representing S.E.M.

During whole-cell recordings, extracellular application of the membrane impermeable physiological reducing agent GSH (10 mM) mimicked the effects

of 10 mM DTT in potentiating the heat-induced membrane currents. The mutation of the three extracellular cysteines (3CYS) fully abrogated the effects of GSH on heat-evoked responses (Fig. 3). These data confirm that sulfhydryl groups facing toward the extracellular side of the membrane are indeed involved in regulating the activity of the TRPV1 channel by reducing agents.

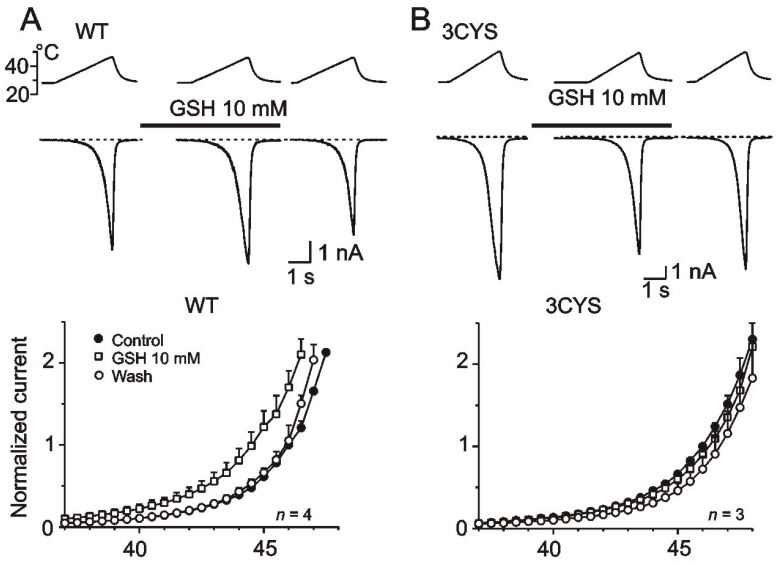

Figure 3. Reducing agent GSH mimics effects of DTT. (A) GSH-potentiated whole-cell currents evoked by temperature ramps in wild-type TRPV1. Current values normalized to currents evoked at 46 °C in extracellular control solution. Averaged currents shown with error bars representing S.E.M. **(B)** GSH failed to affect 3CYS mutant.

Our result demonstrate that membrane-permeable oxidizing compounds such as diamide, chloramine-T and H_2O_2 also strongly potentiate the heat-evoked activity of TRPV1 and that the 3CYS mutation does not influence the effects of these agents (see Fig. 4). The membrane-impermeable cysteine-specific oxidizing agent DTNB has no effect on wild-type TRPV1-mediated heat-induced currents. These results together strongly suggest that there are no oxidizable residues exposed to the surface that may contribute to TRPV1 function. We cannot rule out that the effect of the membrane-permeable oxidizing agents might be a result of alteration in the activity of a protein putatively associated with TRPV1 or the involvement of second messenger signalling cascades. We found that alkylating agent NEM specifically reacts with free sulfhydryl groups, which, in a manner that depends on the preceding treatment with DTT, results in irreversible changes in the activity of the TRPV1 channel. Thus thiol-reactive agents seem to modulate the gating of TRPV1 channels by an action directly on the TRPV1 protein.

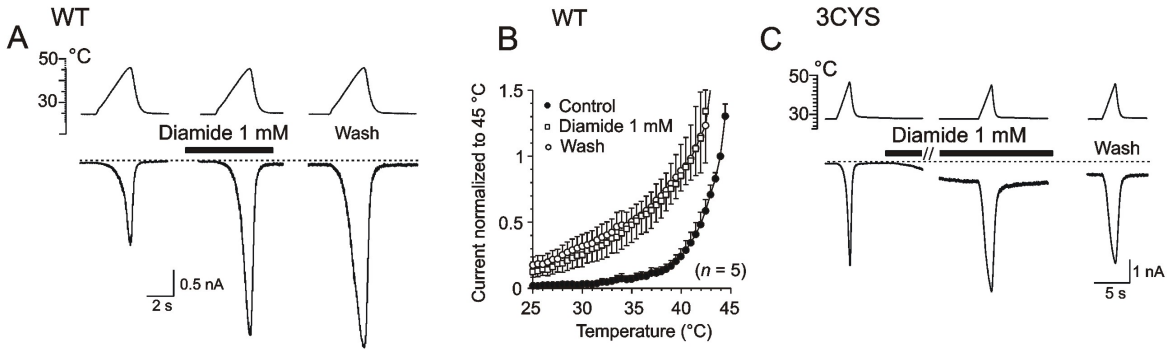

Figure 4. Oxidizing agent diamide irreversibly potentiates TRPV1-mediated heat-induced membrane currents. (A) Effect of diamide on heat-evoked response in HEK293T cell expressing WT-TRPV1. Temperature protocol is shown above. Dashed lines indicate zero membrane current in all records. **(B)** Summary of effects of diamide on heat-evoked currents in five HEK293T cells expressing wild-type TRPV1. Currents normalized to current obtained at 45 °C before application of diamide, and normalized values represent means ± S.E.M. **(C)** Representative heat-induced responses recorded from HEK293T cell transiently transfected with 3CYS mutant of TRPV1.

In this study, we examined the effects of redox-active substances on the heat-induced membrane currents, because increasing the temperature above ~ 42 °C activates the channel directly (Tominaga et al. 1998). This experimental approach has the advantage that no ligand binding to the receptor is needed to gate the channels and direct modification of the ligand molecule by redox-active substances can be avoided. Our data indicate that capsaicin concentration is radically decreased by the presence of oxidizing agents (Fig. 5). More generally, the redox-active substances can substantially affect the activity of TRPV1 channels by influencing their modulators or agonists.

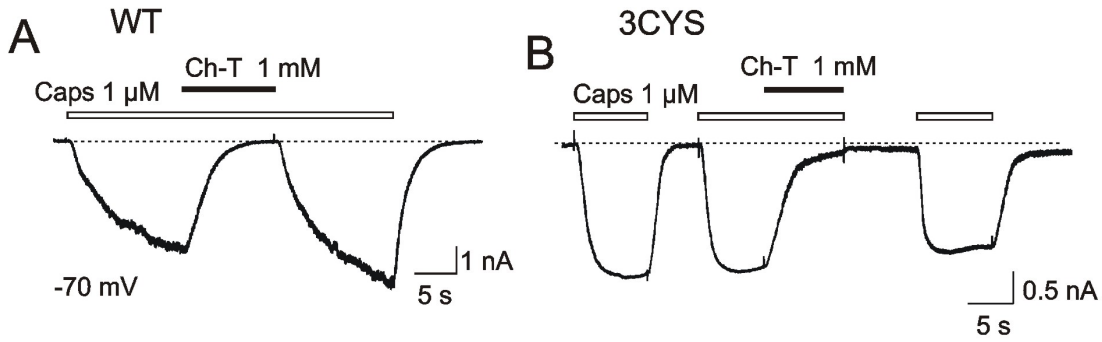

Figure 5. Oxidizing agents influence capsaicin-induced activity of TRPV1 by decreasing its concentration. Representative traces of capsaicin-induced whole-cell responses recorded from HEK293T cell transfected with WT-TRPV1 **(A)** and 3CYS-TRPV1 **(B)** mutant. Capsaicin-induced currents were abolished by coapplication of capsaicin with 1 mM chloramine-T. Bars above the records indicate duration of drug application. The cells were clamped at a membrane potential of -70 mV.

Our studies using various sulfhydryl reagents may help to clarify the multiple mechanisms involved in the modulation of TRPV1 by changes in redox potential. To obtain a more complete picture of the molecular basis of redox modulation, we submitted the TRPV1 protein amino acid sequence from V596 to D654 to the predictive software program DiANNA (DiAminoacid Neural Network Application). The results from the cysteine oxidation state prediction molecule yielded a half-cystine for C621, which was in agreement with our observation that C621 is involved in the modulation of the TRPV1 channel by extracellular DTT. Based on this prediction software, it is likely that there are no intramolecular disulfide bonds within the TRPV1 receptor protein. The process of reduction and oxidation of C621, therefore, may involve an interaction of two adjacent TRPV1 subunits.

5.2. Oxidizing reagent copper-o-phenanthroline is a blocker of the vanilloid receptor TRPV1

Our results demonstrate that oxidizing reagent copper-o-phenanthroline blocks the TRPV1 channel. We showed that the blocking effect of Cu:Phe does not involve extracellularly located cysteines.

We found that Cu:Phe (100:400 μM) produced a profound inhibition of I_{HEAT} to 19.2 ± 4.6% (n = 8) (see Fig. 6*b*). Phenanthroline (400 μM) alone did not have a significant effect on I_{HEAT} (102.8 ± 7.5%; n = 5), while $CuSO_4$ at a concentration of 100 μM increased I_{HEAT} by 44.6 ± 12% at 46 °C (n = 8). The responses to 1 μM capsaicin at room temperature were also effectively blocked by Cu:Phe (100:400 μM) (Fig. 6*d*). We demonstrate that Cu:Phe blocking effect on the capsaicin-induced currents was voltage dependent. Phenanthroline (400 μM) alone caused partial inhibition of the inward currents evoked by capsaicin at negative potentials (by 48.4 ± 12.9% at -70 mV; n = 7), while this inhibition was completely removed at positive potentials. Hundred micromolar $CuSO_4$ applied together with 1 μM capsaicin did not exhibit significant effects on capsaicin-induced currents.

We demonstrate that prolonged pre-exposure (~ 30 s) to Cu:Phe 100:400 did not affect responses induced by low pH; however, it produced a profound inhibition of the membrane currents during application of pH 5 solution. The time constant of inhibition of the low pH-induced currents by Cu:Phe was substantially longer

at the positive membrane potential + 40 mV (τ_{on} = 746 ± 113 ms) compared to that at -70 mV (τ_{on} = 146 ± 27 ms) indicating a pronounced voltage dependence of the channel block (see Fig. 7A). In order to eliminate the possibility that the inhibition could be due to the effects of free copper (II) or the uncomplexed form of o-phenanthroline, control experiments were performed using acidic solution (pH 5) applied with $CuSO_4$ (100 μM) or o-phenanthroline (400 μM). At -70 mV, $CuSO_4$ alone had no significant effects on pH 5-induced response. At a concentration of 400 μM, o-phenanthroline itself inhibited responses elicited by pH 5 (by 36 ± 11% when measured after 10 s of pH 5 application together with 400 μM; n = 6). We assessed the concentration dependence of the inhibition of the responses elicited by pH 5. Based on concentration-response data we calculated an apparent IC_{50} value of 5.2 μM (Cu:Phe = 5.5 : 20.8 μM) and Hill coefficient 1.1 (see Fig. 7B).

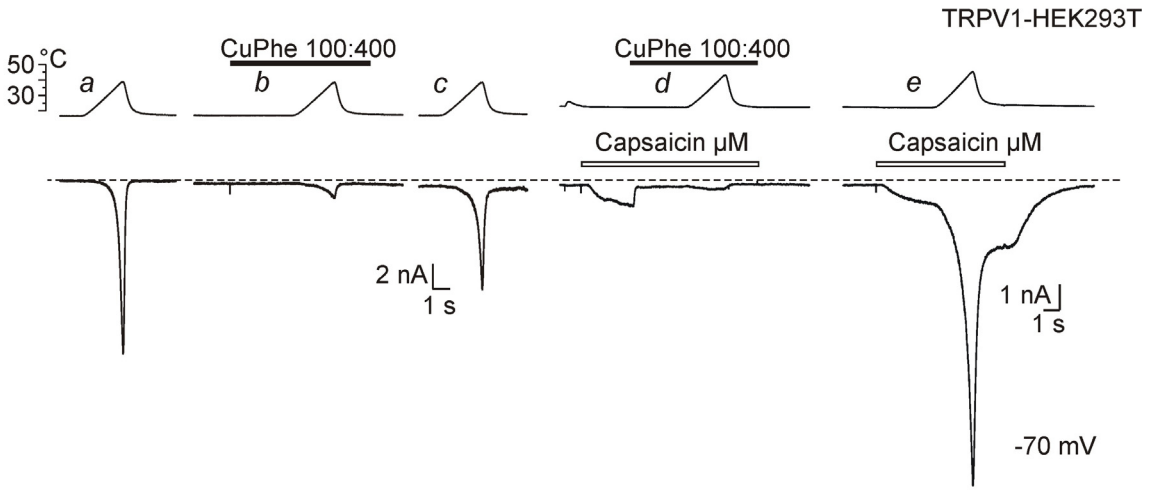

Figure 6. Complex Cu-o-phenanthroline blocks membrane currents induced by noxious heat and capsaicin in TRPV1 transfected HEK 293T cells. Whole cell membrane currents induced by a 3 sec ramps of heated solutions to 47 °C (a) in the control solution, (b) in the presence of Cu-o-phenanthroline 100:400 μM, (c) during wash. (d) The effect of Cu-o-phenanthroline 100:400 μM on the capsaicin-induced response at room temperature and during the 3 sec ramp of elevated temperature to 47 °C. (e) Increase of the heat-induced membrane current in the presence of 1 μM capsaicin recorded 30 s later. The bars above the records show the duration of the drug application. The upper row of the records indicates the temperature of the superfusing solutions.

Our results demonstrate that Cu:Phe blocked the capsaicin- and heat-induced response in DRG neurons to a similar extent as in the TRPV1-HEK293T cells. We also show that Cu:Phe blocked the capsaicin- and heat-induced response in 3CYS-TRPV1- HEK293T cells (see Fig. 8). These findings, together with an almost

complete reversibility of the Cu:Phe blocking effects, excluded the alternative that Cu:Phe inhibits the TRPV1 channel by modifiying the -SH groups of the cysteine residues on the extracellular side of the vanilloid receptor. Given the similarity of TRPV1 molecular architecture to other TRP family members, we demonstrate that TRPM8 receptor can be blocked by Cu-o-phenanthroline. Similarly to TRPV1 the Cu:Phe inhibition was reversible and voltage-dependent.

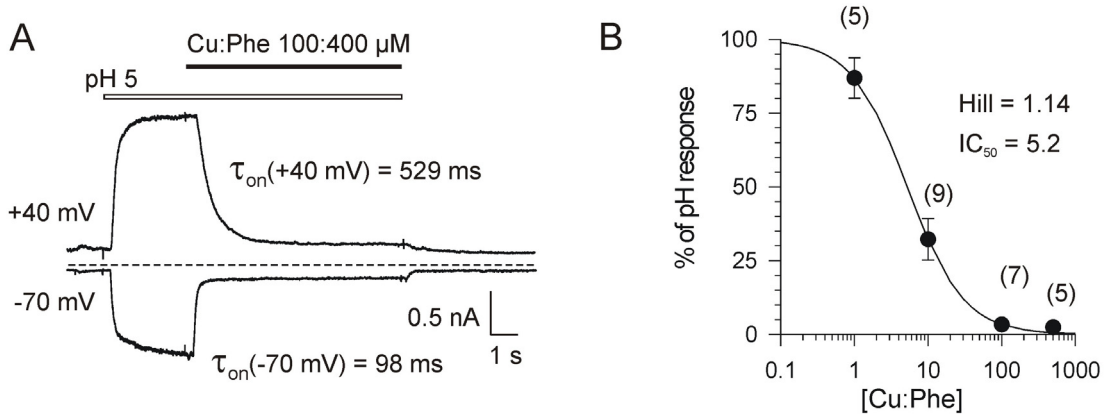

Figure 7. The effect of Cu:Phe on the membrane currents induced by pH 5 in HEK 293T cells expressing wild-type TRPV1. (A) The effect of Cu:Phe on the membrane currents induced by pH 5 at -70 mV and +40 mV. Note the outward rectification of the membrane current induced by pH 5 and the longer time constant of the onset of the inhibition produced by Cu:Phe on the positive membrane potential. The bars above the records indicate the time of drug application. **(B)** Dose response curve of the inhibition of the membrane currents induced by pH 5 produced by Cu:Phe at -70 mV. The number of cells is indicated in brackets.

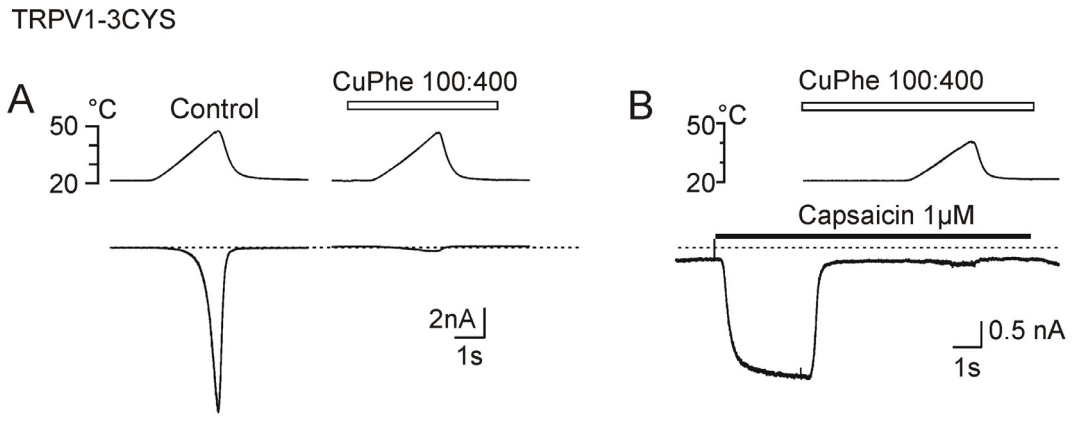

Figure 8. The effect of Cu:Phe 100:400 µM on heat and capsaicin-induced responses in HEK 293T cells transfected with triple-mutant of TRPV1: C616G/C621G/C634G. (A) Whole cell membrane currents induced by a 3 sec ramps of heated solutions to 47 °C in the control solution and in the presence of Cu-o-phenanthroline 100:400 µM. **(B)** The effect of Cu-o-phenanthroline 100:400 µM on the capsaicin-induced response at room temperature and during the 3 sec ramp of increasing temperature to 47 °C (shown above the record).

Our results demonstrate that the complex Cu-o-phenanthroline blocks the TRPV1 channel. The fact that the blocking effect of Cu:Phe is reversible, concentration dependent and does not involve extracellularly located cysteines supported our presumption that a bulky, highly positively charged complex copper-o-phenanthroline enters the extracellular mouth of the channel and blocks it. Cu:Phe can be considered as an additional effective and reversible blocker potentially useful in further investigation of structure-function relationship especially of the pore regions of the TRPV1 and possibly other TRP channels.

5.3. Functional role of C-terminal cytoplasmic tail of the vanilloid receptor TRPV1

C-terminal truncations of rat TRPV1 have been constructed (see Fig. 9) to characterize the contribution of the cytoplasmic C-terminal region to TRPV1 function and to delineate the minimum amount of C tail necessary to form a functional channel. Our results indicate that the cytoplasmic COOH-terminal domain strongly influences the TRPV1 channel activity, and that the distal half of this structural domain confers specific thermal sensitivity.

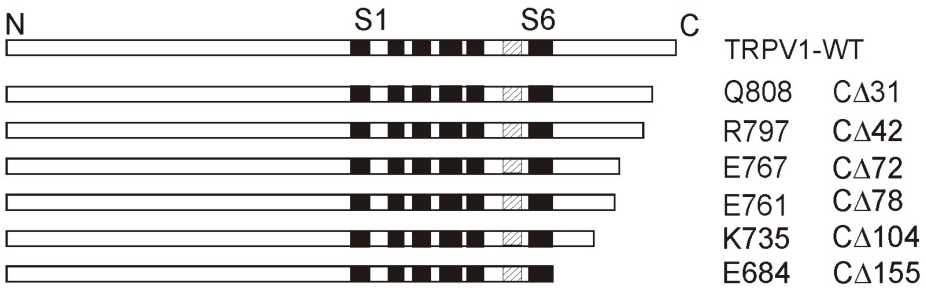

Figure 9. C-terminal truncations of vanilloid receptor TRPV1. Schematic representation of TRPV1 C-terminal deletions showing the six transmembrane-spanning domains, S1-S6, and a pore-forming region (hatched bars).

Deletions of 31 and 42 residues from the C terminal (CΔ31 and CΔ42) did not cause significant changes in capsaicin- and proton-induced maximum currents at room temperature, whereas in mutant receptors CΔ72 and CΔ78, the sensitivities to these two activators were substantially reduced. The CΔ104 mutation displayed no sensitivity to capsaicin over the entire temperature range examined. Sequential deletions of 72, 78,

and 104 residues from the C terminal also reduced the membrane currents elicited by protons; however, responses to pH 5 were still present in all of these mutants (Fig. 10). The thermal sensitivity of TRPV1 mutants was examined using standard ramps of increasing temperature from 25 to 47 °C. In the mutants CΔ31 and CΔ42, the magnitude of I_{HEAT} at 47 °C was not significantly different from that of the wild-type TRPV1. In contrast, sequential deletions of 72, 78, and 104 residues from the C-terminal end reduced the amplitudes of the heat-induced current markedly (see Fig. 10).

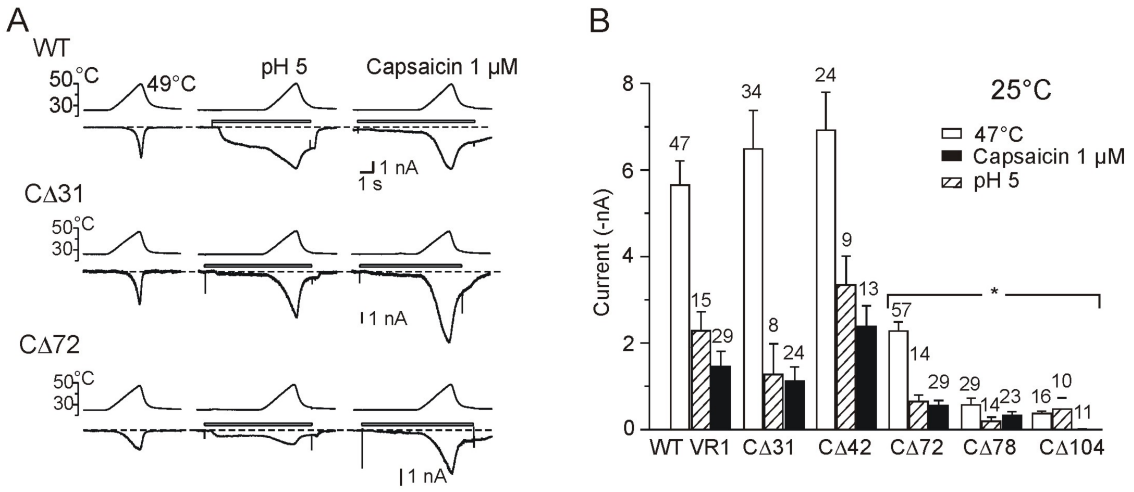

Figure 10. Whole-cell membrane currents induced by heat, protons, and capsaicin in wild-type TRPV1 and C-terminal truncated mutants expressed in HEK293T cells. (A) Heat-evoked currents were induced by a 3 sec ramp of temperature increase to 49 °C in standard extracellular solution (left), acidic pH (pH 5; middle), and capsaicin (1 μM; right). The cells clamped at -70 mV were superfused for the time indicated by the open bars shown above the records. **(B)** Quantitative analysis was performed by first stimulating cells by heat (from 25 to 49 °C) in the extracellular solution, followed by a 30 sec washout, and then by stimulating them by heat in the presence of pH 5 or capsaicin. Mean ± SEM responses evoked by heat, capsaicin, and low pH measured at room temperature, 25 °C, are shown ($p < 0.05$; indicated with an asterisk). The numbers above each bar indicate the number of cells measured.

Sequential deletions of 31, 42, and 72 residues from the C-terminal end also shifted significantly the thermal threshold to 38.6 ± 0.7 °C ($n = 22$), 32.5 ± 0.5 °C ($n = 21$), and 28.6 ± 0.5 °C ($n = 50$), respectively. Deletions of 78 and 104 residues from the C-terminal end shifted the thermal threshold toward temperatures close to that in the bath (Fig. 11). To determine the extent to which the C-terminal tail participates in the regulation of TRPV1 channel activity induced by heat stimulation, the temperature coefficient $Q10$ was estimated for each mutant. Interestingly, the distribution of $Q10$ in the truncated mutant channel lacking the final 31 aa from the C terminal was similar to the wild-type;

however, the activation threshold was decreased significantly. The temperature coefficients were markedly reduced in all of the other mutants examined (see Fig. 11D).

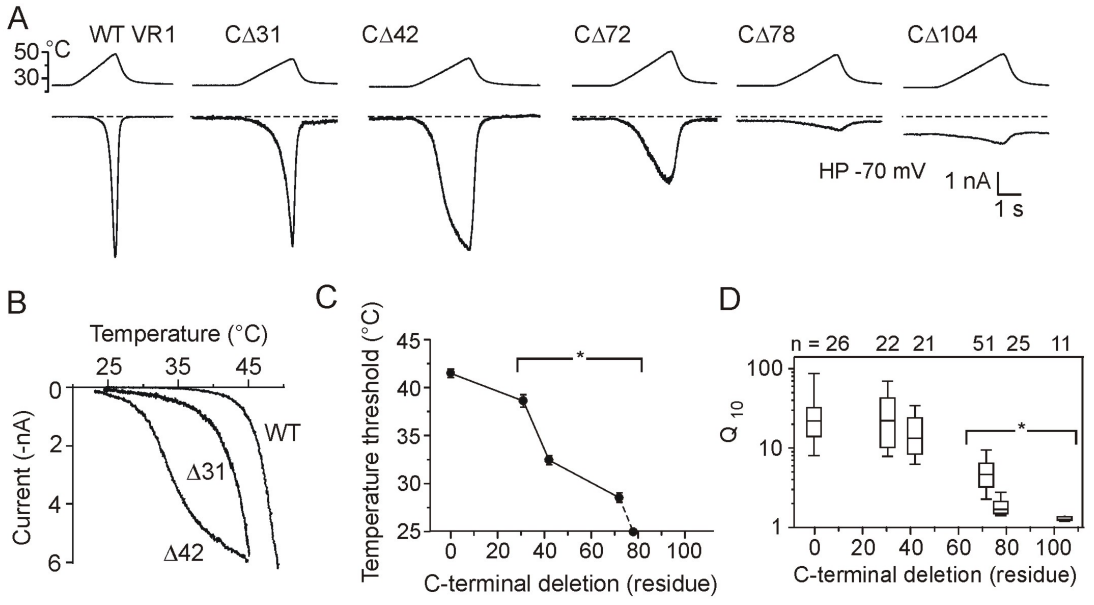

Figure 11. Sensitivity to heat in C-terminally truncated mutant. (A) Representative heat-induced responses recorded from HEK293T cells transiently transfected with wild-type TRPV1, CΔ31, CΔ42, CΔ72, CΔ78, and CΔ104 mutants. Heat-evoked currents were induced by a 3 sec temperature ramp (24-49 °C) in control extracellular solution. **(B)** Temperature response profiles of the heat-induced currents shown in *A* for wild-type, CΔ31, and CΔ42. **(C)** Thermal threshold values plotted versus the number of residues deleted from the C-terminal tail of TRPV1 receptor. Sequential deletions of 31, 42, and 72 residues from the C-terminal end resulted in a significant shift of the thermal threshold to 38.6 ± 0.7 °C (SEM; $n = 22$), 32.5 ± 0.5 °C ($n = 21$), and 28.6 ± 0.5 ($n = 50$), respectively. Deletions of 78 and 104 residues from the C-terminal end caused a shift of the TRPV1 thermal threshold toward temperatures close to that in the bath. **(D)** The median $Q10$ plotted versus the number of residues deleted from the C-terminal tail of TRPV1 receptor. The ends of the boxes define the 25th and 75th percentiles, with a line at the median; error bars define the 10th and 90th percentiles. The asterisk indicates statistically significant difference from the wild-type ($p < 0.05$).

At room temperature and at normal pH 7.3, the rat clone TRPV1 is activated by depolarizing voltages in the absence of any agonists (Reeve and Bevan 2002). To determine the role of the C terminal in voltage-dependent TRPV1 activation, the current-voltage relationships of HEK293T cells transfected with either wild-type or C-terminal truncated TRPV1 were examined. A comparison of the outwardly rectifying currents induced by depolarizing voltage steps to + 80 mV in the wild-type and in the C-terminal truncated TRPV1 constructs shows that there was a decrease in the outward current and a significant slowing of activation and deactivation kinetics as the number of C-terminal amino acid residues deleted was increased (Fig. 12). Mutations

CΔ31 and CΔ42 were less affected, whereas in mutants CΔ72 and CΔ78, the differences in voltage-activated currents were pronounced. A comparison of the current–voltage relationships of the mutants indicates that the current amplitudes at + 80 mV were reduced with shortening of the TRPV1 C terminal, suggesting that the proximal regions of the C-terminal tail were required for efficient voltage gating of TRPV1. The mutant CΔ104 showed no sign of outward rectifying currents, and the leakage current was linearly related to membrane potential. To study the voltage-dependent gating of TRPV1 truncated mutants in more detail, the RIs of the *I–V* relationships for mutant constructs were compared. The RI of the CΔ31 mutant was significantly higher than that of the wild-type receptor (median 9.08 vs 6.42 in wild-type). In contrast, voltage-dependent rectification in the CΔ72 and CΔ78 mutants was significantly reduced (medians, 3.97 and 2.85) (see Fig. 12C).

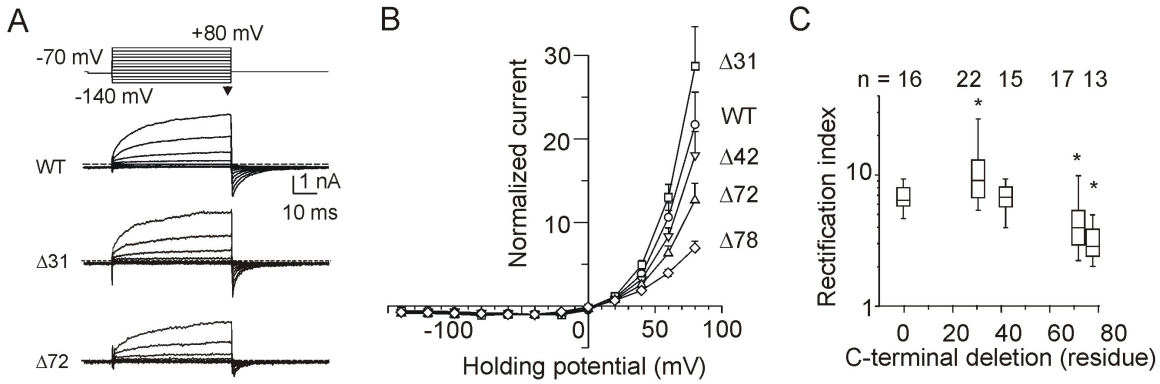

Figure 12. Voltage-activated currents in wild-type TRPV1 and C-terminal truncated mutants. (A) Representative whole-cell membrane currents induced by a sequentially applied series of 50 msec voltage steps ranging from -140 mV to +80 mV, in +20 mV increments. The currents were measured at the end of each pulse (arrow). **(B)** Current-voltage relationship for wild-type and CΔ31, CΔ42, CΔ72, and CΔ78 mutants. Data were normalized to the value of the current at -40 mV. **(C)** For CΔ31, RI was significantly higher than that for wild-type, whereas significantly less RI was observed for CΔ72 and CΔ78 mutants. The ends of the boxes define the 25th and 75th percentiles, with a line at the median; error bars define the 10th and 90th percentiles. The asterisks indicate significant differences from wild-type ($p < 0.05$).

Truncation of the entire TRPV1 C-terminal domain was found to produce a nonfunctional channel. HEK293T cells expressing the C-terminal truncated mutant CΔ155 did not respond to either capsaicin or heat. To explore whether the fully truncated mutant can insert into the cell membrane, we used a polyclonal antibody against the first

21 aa residues of the rat TRPV1 N-terminal end and confocal laser scanning microscopy to compare the surface expression of wild-type and the CΔ155 mutant in transiently transfected HEK293T cells. There were no significant differences between the distribution patterns of anti-TRPV1 immunoreactivity in the wild-type and the CΔ155 mutant (Fig. 13). This assay indicated that the population of truncated construct CΔ155 was processed and not retained in the intracellular compartments, and that the C terminal was not essential for trafficking the receptor to the HEK293T cell surface.

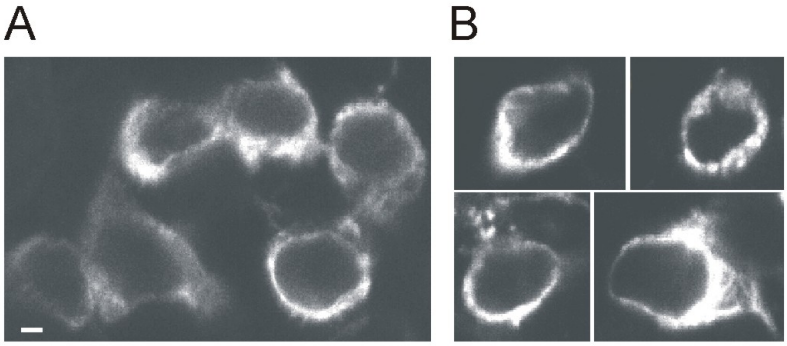

Figure 13. Surface expression of C-terminally truncated TRPV1 construct in transiently transfected HEK293T cells. (A) Confocal microscope image of wild-type transfected HEK293T cells. Permeabilized cells were immunohistochemically labeled using a polyclonal antibody against the first 21 aa residues of the rat TRPV1 N-terminal end. Cells were labeled with FITC-conjugated donkey anti-rabbit IgG secondary antibody and visualized with standard FITC filters. **(B)** Four HEK293T cells transiently transfected with truncated construct lacking the entire C terminal end CΔ155. Scale bar, 5 μm.

To explain and predict the involvement of the C-terminal domain in TRPV1 channel function, the sequence of the TRPV1 C terminal from A690 to K838 was used for homology modeling. This section of the TRPV1 receptor shows a high sequence homology (44%) to the FHIT protein (fragile histidine triad), whose tertiary structure has been solved at 1.85 Å resolution (Lima et al. 1997). The overall predicted structure of the TRPV1 C tail can be described as a general α + β-type. In the molecular model based on homology with the fragile histidine triad protein presented here, the distal most 31 aa residues of the TRPV1 C terminal (Q808-K838) correspond to the α-helical structure H2 and the large flexible loop connecting it with β-sheet 7. Removal of this region is sufficient to shift the thermal threshold for activation from 42 to 39 °C. The effects of the deletion of the remaining 11 loop amino acids in the mutant CΔ42 (R797-K838) suggest the importance of β-sheets 6 and 7 in channel activation. This view is also

supported by construct CΔ72, which lacks β-strands 6 and 7 (E767-K838) and displays profound changes in channel function. The thermal threshold dropped from 41.5 to 28.6 °C, *Q*10 decreased from 25.6 to 4.7, and the currents induced by capsaicin, pH 5, heat, and voltage decreased significantly, suggesting a distinct role of these two β-strands in the C terminal of TRPV1. In the model of the mutant CΔ104, the helix H1 is lost. Accessibility for multimerization in its entire length and its distribution of hydrophobic residues makes it a good candidate for taking part in a multimerization module (see Fig. 14).

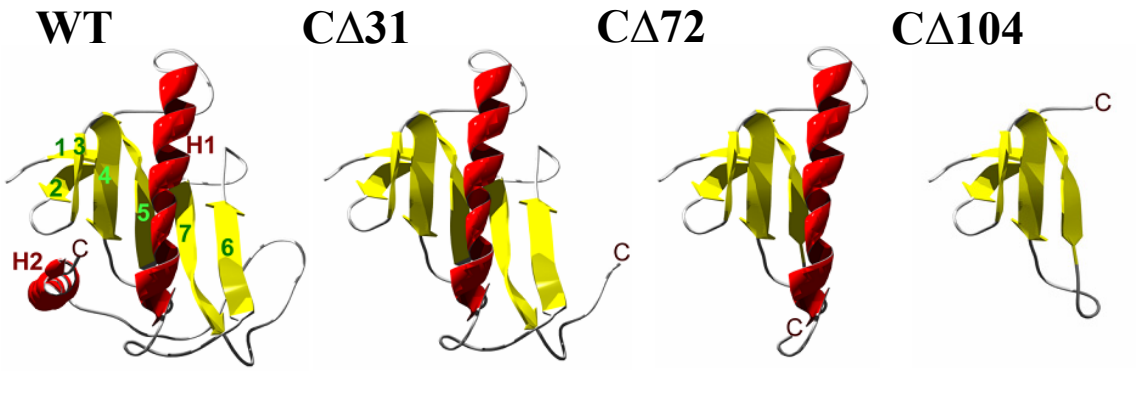

Figure 14. Molecular modeling of the TRPV1 C terminal. Predicted structure of the complete C terminal of TRPV1 and the truncated mutants. **WT**, Ribbon diagram of the wild-type C terminus (residues A690-K838). Homology modeling predicts two α-helices (H1, H2) and seven β-strands (1–7). Antiparallel strands 1 and 2 form a β-hairpin, and strands 3-7 form a five-stranded antiparallel sheet. **CΔ31**, this mutant (residues A690-T807) lacks the α-helix H2. **CΔ72**, in this construct (A690-C766), secondary structural elements H2 and β-strands 6 and 7 are missing. **CΔ104**, the predicted structure of the truncated construct (A690-G734) consists only of β-strands 1–5.

Our results demonstrate that the intracellular C-terminal tail of the vanilloid receptor TRPV1 in HEK293T cells is required for functional channel activation. Sequential deletions of the residues from the C terminal decrease channel sensitivity to capsaicin, low pH, and depolarizing voltage stimuli. However, the most striking effect of shortening the C terminal of TRPV1 was the altered sensitivity to heat. The threshold of heat-evoked currents was progressively shifted to lower temperatures with the decreasing length of the C-terminal tail. Deletions of 31 and 42 aa residues from the C terminal decreased the temperature threshold for channel activation by 3 and 9 °C, with little or no effect on capsaicin or proton responsiveness.

6. Conclusions

1. Our results indicate that reducing and oxidizing agents sensitize heat-activated vanilloid receptor (TRPV1) current. Using site-directed mutagenesis, we identified cysteine 621 as the residue responsible for the extracellular modulation of TRPV1 by reducing agents. We show that increased responsiveness of TRPV1 evoked by oxidizing agents does not involve extracellular cysteines. We also demonstrate that the effects of oxidizing agents on capsaicin-activated TRPV1 channels are caused by a reduction in the concentration of capsaicin rather than by a redox-based mechanism.

2. We demonstrate that the oxidizing reagent copper-o-phenanthroline (Cu:Phe) is a blocker of TRPV1 channel. The inhibiting effect of Cu:Phe is reversible, voltage- and concentration-dependent, and does not involve extracellularly located cysteines.

3. We demonstrate that sequential deletions of the residues from the C terminal decrease TRPV1 channel sensitivity to specific stimuli (capsaicin, low pH, noxious heat, and depolarizing voltage). Particularly, the most prominent effect of shortening the C terminal is the altered sensitivity of TRPV1 to heat. Deletions of 31 and 42 amino acid residues from the C terminal decrease the temperature threshold for channel activation by 3 and 9 °C, with little or no effect on capsaicin or proton responsiveness.

7. References

Alliegro M. C. (2000) Effects of dithiothreitol on protein activity unrelated to thiol-disulfide exchange: for consideration in the analysis of protein function with Cleland's reagent. *Anal Biochem* 282, 102-106.

Caterina M. J., Schumacher M. A., Tominaga M., Rosen T. A., Levine J. D. and Julius D. (1997) The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* 389, 816-824.

Dittert I., Vlachova V., Knotkova H., Vitaskova Z., Vyklicky L., Kress M. and Reeh P. W. (1998) A technique for fast application of heated solutions of different composition to cultured neurones. *J Neurosci Methods* 82, 195-201.

Chuang H. H., Prescott E. D., Kong H., Shields S., Jordt S. E., Basbaum A. I., Chao M. V. and Julius D. (2001) Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P2-mediated inhibition. *Nature* 411, 957-962.

Jahnel R., Dreger M., Gillen C., Bender O., Kurreck J. and Hucho F. (2001) Biochemical characterization of the vanilloid receptor 1 expressed in a dorsal root ganglia derived cell line. *Eur J Biochem* 268, 5489-5496.

Kedei N., Szabo T., Lile J. D., Treanor J. J., Olah Z., Iadarola M. J. and Blumberg P. M. (2001) Analysis of the native quaternary structure of vanilloid receptor 1. *J Biol Chem* 276, 28613-28619.

Kuzhikandathil E. V., Wang H., Szabo T., Morozova N., Blumberg P. M. and Oxford G. S. (2001) Functional analysis of capsaicin receptor (vanilloid receptor subtype 1) multimerization and agonist responsiveness using a dominant negative mutation. *J Neurosci* 21, 8697-8706.

Kwak J., Wang M. H., Hwang S. W., Kim T. Y., Lee S. Y. and Oh U. (2000) Intracellular ATP increases capsaicin-activated channel activity by interacting with nucleotide-binding domains. *J Neurosci* 20, 8298-8304.

Laskowski R. A., MacArthur M. W., Moss D. S. and Thorton J. M. (1993) PROCHECK: a program to check the stereochemical quality of protein structures. *J Appl Crystallogr* 26, 283-291.

Lima C. D., D'Amico K. L., Naday I., Rosenbaum G., Westbrook E. M. and Hendrickson W. A. (1997) MAD analysis of FHIT, a putative human tumor suppressor from the HIT protein family. *Structure* 5, 763-774.

Nilius B. and Voets T. (2005) TRP channels: a TR(I)P through a world of multifunctional cation channels. *Pflugers Arch* 451, 1-10.

Numazaki M., Tominaga T., Toyooka H. and Tominaga M. (2002) Direct phosphorylation of capsaicin receptor VR1 by protein kinase Cepsilon and identification of two target serine residues. *J Biol Chem* 277, 13375-13378.

Pedersen S. F., Owsianik G. and Nilius B. (2005) TRP channels: an overview. *Cell Calcium* 38, 233-252.

Planells-Cases R., Garcia-Sanz N., Morenilla-Palao C. and Ferrer-Montiel A. (2005) Functional aspects and mechanisms of TRPV1 involvement in neurogenic inflammation that leads to thermal hyperalgesia. *Pflugers Arch* 451, 151-159.

Ramsey I. S., Delling M. and Clapham D. E. (2006) An introduction to trp channels. *Annu Rev Physiol* 68, 619-647.

Reeve A. J. and Bevan S. (2002) Voltage-dependent activation of rat cloned vanilloid receptor (VR1) in the absence of exogenous agonist, in *10th World Congress on Pain*. IASP Press, San Diego, CA.

Sali A. and Blundell T. L. (1993) Comparative protein modelling by satisfaction of spatial restraints. *J Mol Biol* 234, 779-815.

Schumacher M. A., Moff I., Sudanagunta S. P. and Levine J. D. (2000a) Molecular cloning of an N-terminal splice variant of the capsaicin receptor. Loss of N-terminal domain suggests functional divergence among capsaicin receptor subtypes. *J Biol Chem* 275, 2756-2762.

Schumacher M. A., Jong B. E., Frey S. L., Sudanagunta S. P., Capra N. F. and Levine J. D. (2000b) The stretch-inactivated channel, a vanilloid receptor variant, is expressed in small-diameter sensory neurons in the rat. *Neurosci Lett* 287, 215-218.

Szallasi A., Lewin N. A. and Blumberg P. M. (1993) Vanilloid (capsaicin) receptor in the rat: positive cooperativity of resiniferatoxin binding and its modulation by reduction and oxidation. *J Pharmacol Exp Ther* 266, 678-683.

Szallasi A., Nilsson S., Farkas-Szallasi T., Blumberg P. M., Hokfelt T. and Lundberg J. M. (1995) Vanilloid (capsaicin) receptors in the rat: distribution in the brain, regional differences in the spinal cord, axonal transport to the periphery, and depletion by systemic vanilloid treatment. *Brain Res* 703, 175-183.

Thompson J. D., Gibson T. J., Plewniak F., Jeanmougin F. and Higgins D. G. (1997) The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 25, 4876-4882.

Tominaga M., Caterina M. J., Malmberg A. B., Rosen T. A., Gilbert H., Skinner K., Raumann B. E., Basbaum A. I. and Julius D. (1998) The cloned capsaicin receptor integrates multiple pain-producing stimuli. *Neuron* 21, 531-543.

Vlachova V., Susankova K., Lyfenko A., Kuffler D. P. and Vyklicky L. (2002) Kapsaicinový receptor: specifický iontový kanál pro detekci bolestivých podnětů. *Psychiatrie* 6, 6-13.

Vyklický L., Vlachová V., Vitasková Z., Dittert I., Kabát M. and Orkand R. K. (1999) Temperature coefficient of membrane currents induced by noxious heat in sensory neurones in the rat. *J Physiol* 517, 181-192.

Xue Q., Yu Y., Trilk S. L., Jong B. E. and Schumacher M. A. (2001) The genomic organization of the gene encoding the vanilloid receptor: evidence for multiple splice variants. *Genomics* 76, 14-20.

8. List of publications:

Published in impacted journals:

1. Vyklicky L, Lyfenko A, Susankova K, Teisinger J, Vlachova V: Reducing agent dithiothreitol facilitates activity of the capsaicin receptor VR-1. Neuroscience 2002;111(3):435-41 (IF = 3.4)

2. Vlachova V, Teisinger J, Susankova K, Lyfenko A, Ettrich R, Vyklicky L: Functional role of C-terminal cytoplasmic tail of rat vanilloid receptor 1. J Neurosci 2003 Feb 15;23(4):1340-50 (IF = 7.5)

3. Tousova K, Susankova K, Teisinger J, Vyklicky L, Vlachova V: Oxidizing reagent copper-o-phenanthroline is an open channel blocker of the vanilloid receptor TRPV1. Neuropharmacology 2004 Aug; 47(2):273-85 (IF = 3.6)

4. Susankova K, Tousova K, Vyklicky L, Teisinger J, Vlachova V: Reducing and Oxidizing Agents Sensitize Heat-Activated Vanilloid Receptor (TRPV1) Current. Mol Pharmacol. 2006 Jul;70(1):383-94 (IF = 4.6)

5. Krusek J, Dittert I, Hendrych T, Hnik P, Horak M, Petrovic M, Sedlacek M, Susankova K, Svobodova L, Tousova K, Ujec E, Vlachova V, Vyklicky L, Vyskocil F, Vyklicky L Jr, Activation and modulation of ligand-gated ion channels. Physiol Res 2004; 53 Suppl 1: S103-13 (IF = 1.8)

6. Tousova K, Vyklicky L, Susankova K, Benedikt J, Vlachova V: Gadolinium activates and sensitizes the vanilloid receptor TRPV1 through the external protonation sites. Mol. Cell. Neurosci. 30 (2005) 207-217 (IF = 4.6)

7. Krumscheid R, Ettrich R, Sovova Z, Susankova K, Lansky Z, Hofbauerova K, Linnertz H, Teisinger J, Amler E, Schoner W: The phosphatase activity of the isolated H4-H5 loop of Na+/K+ ATPase resides outside its ATP binding site. Eur. J. Biochem. 2004 Oct; 271(19):3923-36 (IF = 3.16)

8. Krumscheid R, Susankova K, Ettrich R, Teisinger J, Amler E, Schoner W: Localization of catalytic active sites in the large cytoplasmic domain of Na+/K+-ATPase. Ann N Y Acad Sci 2003 April; 986:242-4 (IF = 1.97)

Other publications:

9. Vlachova V, Susankova K, Lyfenko A., Kuffler DP, Vyklicky L: Kapsaicinovy receptor-specificky iontovy kanal pro detekci bolestivych podnetu. Psychiatrie 2002;6 (Suppl. 4):6-13

10. Vyklicky L Jr, Horak M, Petrovic M, Sedlacek M, Susankova K, Tousova K, Vyklicky L, Vlachova V: Iontove kanaly a modulace jejich aktivity fyzikalnimi a chemickymi podnety. Psychiatrie 2004;8 (Suppl. 3):6-12

11. Vyklicky L, Susankova K, Tousova K, Vlachova V: Vaniloidni receptor TRPV1: aktivace, modulace a uloha v mechanizmech nocicepce. Pohybové ustroji (Pokroky ve vyzkumu, diagnostice a terapii) 2004; Volume 11; Supplementum 1-2; 17-32

12. Susankova K, Vlachova V: Vaniloidni receptor: Struktura jako klic k poznani funkce; Vanilloid receptor: Structure as a key for understanding the function. Bolest 3/2005 111-115